

PARALLEL SCANNED LASER CONFOCAL MICROSCOPE

This invention was made with U.S. Government support under Grant No. DMR-9730189 awarded by the National Science Foundation and through the MRSEC Program of the National Science Foundation under Grant Number DMR-9880595.

The present invention is directed generally to a method and apparatus for creating three-dimensional images of samples using principles of scanned laser confocal microscopy. More particularly, the invention is directed to a method and apparatus for the use of multiple scanned laser beams operating in parallel and in conjunction with a spatially-resolved area detector to receive the optical images created by each of the plurality of beams. This method and apparatus retains all of the advantages of conventional scanned laser confocal microscopy with the substantial additional advantages of (1) greatly increased acquisition speed, (2) the longest possible exposure times for samples which produce low-light-level images, (3) compact design, (4) no moving parts, and (5) the feature of integrated optical trapping with no additional components.

It is known that confocal microscopy can be applied using a tightly focused beam of light to illuminate a sample. The illumination is most intense at the focal point, so that the volume of the sample located at the focal point has more opportunity to scatter the incident light than any other region of the sample. The light detection system in a confocal microscope also is focused onto the same volume of the sample as the illumination system. Light scattered by the sample from this volume thus is preferentially detected relative to light scattered by other regions of the sample. The detection system's selectivity for light scattered within the

illuminated volume typically is enhanced by the addition of one or more apertures which block light emanating from other regions of the sample.

The combination of selective illumination with a focused light source and selective detection with an optical system focused onto the same sample volume (confocal detection) provide a conventional confocal microscope with several capabilities. The confocal detection system's ability to reject light scattered from other regions of the sample makes possible imaging in relatively turbid samples. Confocal imaging with high numerical aperture optics also makes possible imaging with very small depth of focus. Confocal microscopes thus can focus deep into samples and create well-resolved optical slices through a three-dimensional sample with minimal cross-talk or convolution of images between slices. These optical slices then can be combined to create a three-dimensional representation of the sample.

The principal practical considerations for establishing confocal microscopy are (1) to scan the focused illumination through the sample in a desired pattern and (2) to maintain confocal detection by keeping the focal volume aligned with the illumination volume. A typical conventional implementation of laser scanning confocal microscopy 128 is shown in FIG. 1. A collimated laser beam 100 passes through a beam splitter 110 before being deflected by a gimbal-mounted mirror 114, or equivalent beam steering device. This beam is directed into the back aperture of the microscope's objective lens 125 through a relay lens consisting of lenses 115 and 116 and beam splitter 120. Typically, an objective lens 125 and the beam splitter 120 are included in the body of the conventional optical microscope 128 and the additional components are mounted outside the microscope's body as a separate assembly. The laser beam 100 is focused to a point 140 by the objective lens 125 to illuminate a sample 142,

and 144 light is scattered by the sample 142, and light 144 radiates in all directions. Some fraction of this scattered light 144 falls within the acceptance solid angle of the objective lens 125 and travels backwards down the beam line followed by the illumination light 100. This fraction is labeled 101 in FIG. 1 and is shown superimposed on the illuminating beam 100. The returned beam 101 emanates from the focal point 140 of the objective lens and so is collimated by the objective lens. Light originating from other sources (not shown in FIG. 1) is not collimated by the objective lens. In practice, both the illuminating 100 and returned 101 beams would fill the entire aperture of the optical train. The returned beam 101 is reflected by the gimbal mounted mirror 114 back along the path taken by the illuminating laser 100 and then reflected again by beam splitter 110. The collimated returned beam passes through aperture 118 and is detected by photodetector 119. Rays of light emanating from a source not located at the focus 140 would not pass through aperture 118 and so would not be detected.

Tilting the gimbal-mounted mirror 114 deflects the illuminating beam 100 and so translates the focal spot 140 across the microscope's field of view. Because the returned beam 101 follows the same optical path as the illuminating beam, up to the beam splitter 110, the detection system is confocal with the illumination system. Scanning the focal spot 140 across the field of view with the gimbal-mounted mirror 114 and correlating the signal measured with the photodetector 119 with the mirror's deflection angle yields a two-dimensional optical slice through the sample 142.

The beam splitters 110 can be selected to optimize illumination and detection efficiency. If the returned beam has the same wavelength as the illumination beam, efficiency could be improved by using a polarization selective form of the beam splitter 110 and adding

polarization-rotating components in the beam line. If the returned beam has a different wavelength because it results from fluorescence, for example, then selection could be based on wavelength, using a dichroic form of the beam splitter 110.

The rate at which such an optical slice can be obtained is limited by the rate at which the beam can be deflected by mirror 114. A mechanical deflector, such as a gimbal-mounted form of the mirror 114, offers a relatively slow deflection rate, with a bandwidth typically well below 1 kHz. Acousto-optical and electro-optical deflectors offer much higher bandwidths but introduce aberrations into both the illuminating and returned beams whose severity varies with the deflection angle. Increasing the deflection rate to increase the imaging rate has the undesirable consequence of reducing the length of time that the illuminating beam is focused on any particular region of the sample. Imaging weakly scattering samples therefore, is hampered by low light levels (and thus low contrast) at the detector 119. A number of disadvantages therefore exist for a conventional single beam confocal microscopy system.

SUMMARY OF THE INVENTION

Parallel laser scanning confocal microscopy uses a plurality of laser beams to scan through a sample simultaneously, and a pixellated area detector is preferably used to detect separately the light scattered by each of the plural laser beams. Scanning a plurality of laser beams through the sample simultaneously provides several advantages over conventional single-beam scanning laser confocal microscopy. For equal scanning rates, parallel scanning reduces the total data acquisition time for one slice by a factor equal to the number of beams. This can be useful for high-speed imaging of moving samples. Further improvements in simultaneous imaging accrue from having many beams probe many regions of the sample

simultaneously. Single-beam systems, by contrast, expose one volume element at a time, so that the last volume element is imaged one entire scan period after the first volume element.

For equal acquisition times, parallel scanning increases the illumination period for each volume of the sample by a factor equal to the number of beams. This can be extremely useful for weakly-scattering samples by permitting much longer exposure times without increasing the time to acquire a complete image. Furthermore, delicate samples can be imaged in proportionately lower light levels, thereby reducing the possibility of damage by laser irradiation.

Other important advantages are that parallel laser scanning confocal microscopy can be implemented with fewer optical components and without moving parts. The simplified optical train can be aligned simply and precise alignment can be obtained automatically under software control, thereby relaxing the specifications on alignment and alignment stability during manufacturing.

It is therefore an object of the invention to provide an improved method and system for confocal microscopy.

It is another object of the invention to provide an improved method and system for plural beam laser scanning microscopy.

It is a further object of the invention to provide an improved method and system for parallel laser beam scanning confocal microscopy.

It is an additional object to provide an improved method and system for plural laser beam scanning of weakly scattering and light sensitive samples for enhanced image formation without sample damage.

Other objects, advantages and features of the present invention will be readily apparent from the following description of the preferred embodiments taken in conjunction with the accompanying drawings described hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates a conventional laser scanned confocal microscopy system;

FIG. 2 illustrates one embodiment of a confocal microscopy system of the invention;

FIG. 3 illustrates optical system rejection characteristic of the system of FIG. 2; and

FIG. 4 illustrates another embodiment of a confocal microscopy system of the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

One embodiment of a parallel laser scanning confocal microscope is shown generally at 200 in FIG. 2. A collimated laser beam 202 is incident on a diffractive medium, such as a diffractive beam splitter 204 which divides the beam 202 into N separate collimated beams, only one beam 208 of which is shown in FIG. 2 for clarity. Each of the diffracted beams 208 emanates from the diffractive beam splitter 204 at a distinct direction denoted by the solid angle Ω . The number N of the beams 208, their relative intensities, and their angular configuration Ω , all are most preferably determined by a computer-generated hologram encoded in the diffractive beam splitter 204. This diffractive beam splitter 204 can be

implemented with a variety of media including addressable liquid crystal phase shifting arrays, microelectromechanical (MEMS) micromirror arrays, or diffractive optical elements encoded in the surface relief or dielectric constant of otherwise transparent substrates, or encoded in the surface of reflective surfaces. Such systems are represented as 400 in FIG. 2.

Each of the diffracted beams 208 is transferred by a relay lens system, one embodiment of which includes a first lens 210 and a second lens 212, also shown in FIG. 2. The lenses 210 and 212, or equivalent optical elements, are arranged so that a collimated beam of light, such as 208, emerging from the center of the diffractive beam splitter 204 also passes as a collimated beam 208 through the center of the entrance pupil of focusing element 214. In a preferred embodiment, this focusing element 214 consists of a high-numerical aperture objective lens. In the implementation depicted in FIG. 2, the beams 208 are reflected into the back aperture of the focusing element 214 by a beam splitter 204 whose reflective properties are chosen to direct the illuminating laser light towards the focusing element. Each collimated one of the beams 208 enters the back aperture of the objective lens (the focusing element 214) at a distinct angle which is proportional to the angle Ω , which is established by the diffractive beam splitter 204. Thus, each of the beams 208 comes to a separate focus in the focal plane of the objective lens 214 at a displacement from the center of the field of view proportional to Ω . By controlling the number N and direction Ω of the beams 208 created from the collimated laser beam 202, the diffractive beam splitter 204 controls the pattern and location of focused spots of laser light in its object plane. The particular focal spot for the beam 208 is indicated at 224 in FIG. 2.

Some of the light from the beam 208 focused at a focal point 224 will be scattered by sample 216 at that focal point 224. This light component emanates as if from a point source in the focal plane of the objective lens 214 and will be collimated thereby, returning down the optical path initially taken by the illuminating collimated beam 208. Rather than allowing this returned light to travel all the way down the illumination path, as shown in the prior art system of FIG. 1, the second beam splitter 218 allows a returned beam 220 to pass through to the microscope's imaging optics, represented schematically by an ocular lens 222 in FIG. 2. The returned beam 220 is shown superimposed on the collimated beam 208 for clarity. In practice, both the beams 208 and 220 would fill the aperture of the objective lens 214.

Each of the collimated beams 208 created by the diffractive beam splitter 204 illuminates a separate volume of the sample 216 and thus results in a separate returned beam, such as the returned beam 220 resulting from one of the collimated beams 208. The intensity of each of these returned beams 220 depends on the efficiency with which each region of the sample 216 scatters laser light. Each of the returned beams 220 is brought to a separate focus by the ocular lens 222, with only the particular focus for the returned beam 220 being shown in FIG. 2 for clarity, with the focal point being indicated at 224.

The individually focused beams of light from the returned beams 220 can be detected simultaneously with a pixellated area detector 226, such as a charge-coupled device (CCD) camera or other numerous conventional area sensor technologies available to detect light at selected locations. These technologies include but are not limited to photodetector arrays, microchannel plates, and complementary metal-oxide-semiconductor (CMOS) detectors. The location \vec{r} , of one of the particular returned beams 220 on the detector 226 depends on the

direction Ω at which the collimated beam 208 was created by the diffractive beam splitter 204. The angular range Ω can be selected so that \vec{r} coincides with one of the pixels on the area detector 226 for each of the N illuminating collimated beams 208. This alignment can be obtained by calculating approximately the phase shifting pattern projected by the beam splitter 204 and can be considered as virtual alignment. Virtual alignment can be obtained under software control by imaging a uniformly reflective surface and calculating holograms which project spots centered on pixels located in the area detector 226.

If the computer-generated diffractive beam splitter 204 is implemented in the form of an addressable device, such as a spatial light modulator, then the beam configuration can be updated with a new pattern, thereby addressing a new set of sample volumes whose images will be projected onto a new set of pixels on the area detector 226. In this way, one optical slice of the sample 216 can be scanned by updating the diffractive beam splitter 204 with a sequence of complementary patterns.

Furthermore, the embodiment of the invention in FIG. 2 indicates that beam splitter 204 operates in a transmission mode. The same basic scheme will operate also with a reflective diffractive form of the beam splitter 204, with appropriate modifications being made in the optical train. One form of this embodiment will be described hereinafter as shown in FIG. 4 as one example of the reflective mode of operation.

Another advantage of the microscope 200 as depicted in FIG. 2 is the lack of any apertures, unlike the prior art design in FIG. 1. Although there are no apertures, the microscope 200 still achieves excellent confocal imaging. Consider a region of the sample 216

near, but not at the confocally illuminated volume disposed about the focal point 224 in FIG.

2. An example of such a location is denoted as region 228 in FIG. 3. Some of the light scattered by this region 228 will be collected by the objective lens 214. However, because this source of light (the region 228) does not lie in the objective's focal plane, the returned light 230 is not collimated. Rather than being brought to a focus by the ocular lens 222 onto the area detector 226, the returned light 230 is defocused. This diffuse scattering pattern, labeled as zone 232 in FIG. 3, delivers much less light to the pixel at \vec{r} than would an equivalent element of the sample 216 at the confocal focal point 224. This intensity reduction comes from two sources. In the first case, the illumination is far less intense away from any of the confocal points, than it is at the confocal focal point 224. Thus, there is less light to scatter at non-confocal points. In fact, the sources of detectable image light must come from the intensely illuminated regions near the confocal points. The returned fraction of the non-confocal scattering then is further reduced in intensity by being spread across several detector pixels of the area detector 226 other than the confocally illuminated pixel at position \vec{r} .

Each confocally illuminated pixel of the area detector 226 therefore is surrounded by a "zone of confusion" (the zone 232) of approximate radius δ within which non-confocal regions of the sample 216 contribute to the detected signal. This light would be filtered out in a standard confocal optical train such as in the prior art embodiment of FIG. 1 by an aperture 118. This light can be rejected in the microscope 200 by ignoring the data generated by pixels in the zone 232 around each confocally illuminated pixel of the area detector 226. Rejecting signals from non-confocally-illuminated pixels performs the task normally performed by an aperture 118 and thus can be functionally considered a virtual or synthetic aperture.

If the beam splitter 204 of FIG. 2 produces the collimated beams 208 whose images were closer than δ on the area detector 226, then non-confocal scattering from each would be detected by the others, thereby degrading performance. The pattern of the beams 208 created by the beam splitter 204 therefore is most preferably chosen so that no two images are closer than δ at the area detector 226. Minimizing crosstalk between simultaneously illuminated pixels of the area detector 226 in this manner sets the maximum number N of spots which can be used to illuminate the sample 216 in any configuration. If the area detector 226 has M pixels, then $N \approx M / (4\delta^2)$.

It should be noted that the confocal microscope 200 can also be adapted to function in an optical tweezer mode. This additional use can be accomplished by increasing the intensity of light to one of the illuminating collimated beams 208 enabling function as an optical tweezer. Varying the intensity of one or more beams relative to the others can be accomplished by computing and projecting an appropriate diffraction pattern in which the desired trapping beams receive a greater proportion of the light available in the beam 202. This operation can be performed in tandem with varying the power of the laser beam 202 so as to maintain constant imaging intensity during trapping. This optical tweezer mode of the microscope 200 also could operate to provide a converging or diverging light beam 208 which would be brought to a focus on form an optical trap out of the focal plane of the objective lens 214, provided an appropriate hologram were computed, and thus the light scattered by the trapped portion of the sample need not be detected by the confocal detection scheme, unless so desired.

002
003
004
005
006
007
008
009
010
011
012
013
014
015
016
017
018
019
020
021
022
023
024
025
026
027
028
029
030
031
032
033
034
035
036
037
038
039
040
041
042
043
044
045
046
047
048
049
050
051
052
053
054
055
056
057
058
059
060
061
062
063
064
065
066
067
068
069
070
071
072
073
074
075
076
077
078
079
080
081
082
083
084
085
086
087
088
089
090
091
092
093
094
095
096
097
098
099
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000

In yet another example form of the invention shown in FIG. 4 a parallel scanned confocal microscopy system 300 employs a reflection-mode spatial light modulator 302. A beam of light 304 is incident on the face of the spatial light modulator 302 (hereinafter SLM 302). The SLM 302 encodes a phase modulation on the beam of light 304 suitable for splitting the beam of light 304 into several independent beams, only one of which 304 is shown for clarity. Each of the beams of light 304 is directed by the same phase pattern into a distinct direction, with the depicted collimated beam 304 being directed at solid angle Ω away from an optical axis 306. Each of the collimated beams 304 created and directed by the phase pattern of the SLM 302 is transferred to the back aperture of the objective lens 214 (or other suitable focusing optical element) to create the diffraction limited focal point 224. In FIG. 1 the collimated beams 304 are transferred with two lenses 308 and 310 arranged to create a plane conjugate to the objective's back aperture at the center of the SLM 302. The optical axis 306 is thus established so that a beam of light passing from the SLM 302 along the optical axis 306 will pass through the center of the objective's back aperture and come to a focus in the middle of the objective's focal plane. A beam such as the collimated beam 304 traveling at an angle of Ω with respect to this optical axis 306 passes through the middle of the back aperture at an angle and thus forms the focal point 224 away from the center of the focal plane. The beam splitter 218 serves to direct the collimated beams 304 into the aperture of the objective lens 214.

Any material at the focal point 224 can scatter some of the incident light out of the focal point 224. Some of this scattered light can be collected by the objective lens 214 to form a returned beam 220. The second beam splitter 218 can be selected to transfer some or all of

this returned beam 220 to the imaging microscopy system 300 and the area detector 226. Light emanating from the focal point 224 is focused by the ocular lens 222 into a spot on the area detector 226 centered at position 312. This position 312, in turn, depends on the angle Ω that the illuminating collimated beam 304 makes with the optical axis 306. This, in turn depends on the phase pattern encoded in the SLM 302.

In regard to resolution of positioning the collimated beam of light 304, a typical form of the SLM 302, has a square or rectangular array of phase-shifting pixels, each of which typically covers a square or rectangular region of the SLM's active aperture. If the SLM 302 has N pixels in one dimension, and each pixel can implement p levels of phase shift, ranging between 0 and 2π radians, then the SLM 302 can steer a beam into $2Np$ directions along that dimension. The actual angular deflection depends on the separation between pixels a and the wavelength of light λ , with the increment between angular deflections being $\lambda / (Npa)$ in the paraxial approximation. The same result obtains for the SLM 302 or diffractive beam splitter 204 operating in reflective or transmissive mode.

The resolution with which the collimated beams 304 directed by the SLM 302 can be positioned on the area detector 226 depends on the magnification of the microscopy system 300, shown schematically as the simple ocular lens 222 in FIG. 4, and on the number M of detector pixels in a given dimension. The optimal magnification matches the scan range obtained from the SLM 302 with the active area of the area detector 226. In this condition, an individual one of the collimated beams 304 can be placed to within $M / (Np)$ of the center of an imaging pixel. Alignment accuracy approaching 1/10 pixel therefore can be obtained over a typical 512×512 imaging area using commercially available ones of the SLM 302.

While preferred embodiments of the invention have been shown and described, it will be clear to those skilled in the art that various changes and modifications can be made without departing from the invention in its broader aspects as set forth in the claims provided hereinafter.

011.365007.1